EXHIBIT 12

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Real-time monitoring of non-viable airborne particles correlates with airborne colonies and represents an acceptable surrogate for daily assessment of cell-processing cleanroom performance

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Abstract

Background aims—Airborne particulate monitoring is mandated as a component of good manufacturing practice. We present a procedure developed to monitor and interpret airborne particulates in an International Organization for Standardization (ISO) class 7 cleanroom used for the cell processing of Section 351 and Section 361 products.

Methods—We collected paired viable and non-viable airborne particle data over a period of 1 year in locations chosen to provide a range of air quality. We used receiver operator characteristic (ROC) analysis to determine empirically the relationship between non-viable and viable airborne particle counts.

Results—Viable and non-viable particles were well-correlated ($r^2 = 0.78$), with outlier observations at the low end of the scale (non-viable particles without detectable airborne colonies). ROC analysis predicted viable counts ≥0.5/feet^3 (a limit set by the United States Pharmacopeia) at an action limit of ≥32 000 particles (≥0.5 μ)/feet^3, with 95.6% sensitivity and 50% specificity. This limit was exceeded 2.6 times during 18 months of retrospective daily cleanroom data (an expected false alarm rate of 1.3 times/year). After implementing this action

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limit, we were alerted in real time to an air-handling failure undetected by our hospital facilities management.

Conclusions—A rational action limit for non-viable particles was determined based on the correlation with airborne colonies. Reaching or exceeding the action limit of 32 000 non-viable particles/feet ^{3} triggers suspension of cleanroom cell-processing activities, deep cleaning, investigation of air handling, and a deviation management process. Our full procedure for particle monitoring is available as an online supplement.

Keywords

airborne colonies; airborne particulates; cleanroom; current good manufacturing practices; environmental monitoring

Introduction

Hematopoietic stem cell transplantation is the oldest and most widely practiced form of regenerative cellular therapy, with more than 16 000 transplants in 2011 in the USA alone (1). As such, it remains a model for the widespread application of emerging cellular therapies. For both historical and regulatory reasons, most products used in blood and bone marrow transplantation are not regulated by the USA Food and Drug Administration (FDA) under current good manufacturing practices (cGMP), a set of rules initially developed for the manufacture of conventional pharmaceuticals and later applied to human cell and tissue products. Despite the exemption from cGMP, centers performing blood and bone marrow transplantation in the USA are increasingly undergoing voluntary certification by the Foundation for Accreditation of Cellular Therapy (FACT), and FACT standards have been converging with cGMP with each successive revision. In contrast with minimally manipulated blood and bone marrow products, cellular therapies involving more than minimal manipulation (e.g. cell separation, culture and genetic modification), termed Section 351 products, must comply with cGMP, as described in subparts B, C and D of 21 Code of Federal Regulations (CFR) 1271. These regulations include a provision for environmental control and monitoring under 21 CFR 1271.195 and 21 CFR 211.42 (2,3). However, the interpretation and implementation of these provisions is left largely to the individual laboratory.

Airborne particulate content, a metric of air quality, can be classified as viable or non-viable contaminants and may originate from humans (e.g. skin cells) (4 – 7) or from areas of production (e.g. opening packaging materials and operating centrifuges or vortexes) (4,8). These contaminants have been implicated in adverse effects on patient health (9 – 12), justifying the need for monitoring airborne particulate concentrations. While there are numerous sources of information regarding metrics, methods and corrective actions for air-quality monitoring in cellular processing facilities (13 – 16), no specific recommendations are available for interpreting non-viable particle counts in the context of airborne microbial contamination.

We sought to implement and validate an environmental monitoring protocol that would allow for real-time evaluation of air quality on a routine basis and help predict microbial

contamination in the cleanroom environment of our cellular therapy laboratory. We tested the hypothesis that non-viable particulate counts can be used to predict viable particulate counts in the maintenance of International Organization for Standardization (ISO) class 7 conditions, and attempted to specify action limits for which we could provide a quantitative rationale.

Methods

Air sampling

This study was performed at the University of Pittsburgh Medical Center Hematopoietic Stem Cell Laboratory (UPMC HSCLab; Pittsburgh, PA, USA), a FACT-accredited good manufacturing process (GMP)-compliant facility equipped with an ISO class 7 cleanroom. Twenty paired air sample measurements from each of five unique locations within the laboratory were analyzed for both non-viable and viable particulate counts over a 1-year period. Additional daily non-viable particle count data were collected over the preceding 18 months. Air-sample locations were chosen to provide a wide range of particle counts and included: the corridor (Figure 1H), testing laboratory (Figure 1T), anteroom between testing laboratory and cleanroom (Figure 1A), ISO class 7 cleanroom by the sample pass-through (Figure 1C and V) and the class II biologic safety cabinet within the cleanroom (Figure 1B). Non-viable particles were counted with an HACH Ultra Met One 227B airborne laser particle counter (Hach Ultra Analytics Inc., Grants Pass, OR, USA). Particle counts were obtained in 10 replicates of 1 min at each location for particle sizes of ≥0.3 and ≥0.5 microns. Data were transferred to a SYSTAT 12.0 datafile (SYSTAT Inc., Chicago, IL, USA). The first two replicates were deleted to eliminate sample carryover, and the remaining eight samples were averaged. The results were graphed for each location as Levey – Jennings plots. Viable particles (bacterial and fungal colonies) were sampled from 1000 L of air over 10 min with a Biotest HYCON RCS high-flow microbial airborne sampler (Biotest Diagnostics Corporation, Dreieich, Germany), using TCI- γ casein soybean digest agar strips (catalog number 941125; Biotest). Cultures were held at 37 ° C in ambient air and read visually for colony counts after 3 and 5 days in culture. Instruments are calibrated yearly by the manufacturers, and were in calibration during this study.

Speciation of organisms

Viable and non-viable particle counts were performed by an independent testing group (Filtech Inc., West Homestead, PA, USA) and speciated by US Micro-Solutions (Greensburg, PA, USA) during certification of the laboratory heating, ventilation and air-conditioning (HVAC) system and biologic safety cabinets, which were staggered at quarterly intervals.

Statistics

All particle count data were converted to particles per cubic foot of air. Particle data were right skewed and required log transformation prior to analysis. Zero counts were replaced with 0.1 prior to log transformation. Graphics and Levey – Jennings plots were created in SYSTAT, as was the receiver operating characteristic (ROC) analysis. A colony count ≥0.5 colonies/feet ^{3} (*c.* 20 colonies/m ^{3}) was used as a cut-off point for the true state in ROC

analysis, based on United States Pharmacopeia (USP) recommendations for ISO class 7 cleanrooms (17), as well as inspection of the historical distribution of colony counts in our cleanroom. Response bins were created in half-log $_{10}$ steps, with two additional intermediate log-spaced bins where data were most dense.

Retrospective analysis to determine the action limit frequency

Eighteen months of daily (Monday – Friday) retrospective non-viable particle count data from the same laboratory were also collected for analysis and to determine the frequency with which action limits would be triggered.

Results

Non-viable particle counts and viable colony counts

Viable microbial colony counts and non-viable laser particle counts were compared by taking 20 paired air-sample measurements collected in five different locations in the laboratory (Figure 1). Non-viable particle counts (≥0.5 microns) ranged from 0 (biologic safety cabinet; Figure 1B) to 1×10^7 (hallway-Figure 1H) particles/feet ^{3} air (Figure 2A). Viable counts after 3 days of incubation ranged from 0 to 2.5/feet ^{3} air (Figure 2B). Linear regression analysis demonstrated a strong relationship between non-viable and viable counts taken from all sites (Figure 3; $r^2 = 0.78$). However, examination of the regression residuals revealed a bias at the low end of the scale, where non-viable particles were detected in the absence of viable particles. These events occurred exclusively in the cleanroom (Figure 3C and V).

ROC analysis

In order to determine whether non-viable particle counts could serve as a practical surrogate for colony counts, ROC analysis was performed on the data generated from the two different analyzers (Figure 4). Consistent with USP recommendations for ISO class 7 cleanrooms (17), 0.5 colonies/feet ^{3} was utilized as a cut-off point for the true state, i.e. the concentration of airborne microbes that should not be exceeded. Based on analysis of the sensitivity and specificity at various points on the ROC curve, 32 000 non-viable particles/ feet ^{3} was established as the action limit. This limit translated to a 95.6% sensitivity (proportion of true contaminants detected; Figure 4, *y* -axis) and 50.0% specificity (100-false alarm rate; Figure 4, *x* -axis); 95.6% sensitivity can be interpreted to mean that, given an action limit of 32 000 particles/feet ^{3} , we would have a 95.6% probability of detecting a true contaminant, should it be present. A false alarm rate of 50% means that for half of the time that we reached the action limit for non-viable particles, no contaminant would be present.

Expected false positive frequency

In order to determine whether a false-positive rate of 50% would be acceptable, we examined 18 months of retrospective non-viable particle data drawn from site 4 in the ISO class 7 cleanroom (Figure 5). Three-hundred and eighty-three air samples were analyzed in eight replicates during this time period, and the upper 99th percentile of these data was determined to be 33 778 non-viable particles/feet ^{3} , a value very close to the action limit (32 000 non-viable particles/feet ^{3}) as determined by ROC analysis. Thus, during 1 year of data

collection (approximately 260 observations), we would expect to meet or exceed the action limit once per 100 observations, or 2.6 times. Assuming a 50.0% false-positive rate, this would have resulted in 1.3 instances per year when corrective action in response to possible contamination would have been unnecessary.

Performance during an air-handling failure

Shortly after instituting a new particle monitoring procedure based on the results presented above, abnormally high particle counts (100 000 particles/ feet ^{3}) were detected in the cleanroom (Figure 1C) during routine real-time monitoring (Figure 6). Hospital plant maintenance was contacted immediately to determine the source of the increased particulates. It was determined that plant maintenance had conducted a test of the emergency power system during the previous night during which power was momentarily lost. Unknown to them, the dedicated air-handling system for the laboratory had failed to restart properly. Further, the local air pressure differential alarm in the Hematopoietic Stem Cell (HSC) laboratory failed to alert because of an incorrect set-up. A deep cleaning was conducted according to our laboratory procedure after the system was reset, and particle counts returned to within normal limits. At no time were particles detected in the class II biosafety cabinet located in the affected cleanroom (Figure 6).

Discussion

The goal of particulate monitoring is to demonstrate that a cleanroom is operating within a consistent state of control and not that the environment is sterile. Under both 21 CFR Part 1271.195 and 21 CFR 211.42 (2,3), monitoring and maintenance of air filtration is required for cGMP compliance. How this monitoring is performed and how particle data are used is left largely to the discretion of the laboratory.

Cleanroom standards were originally defined under the now obsolete Federal Standard 209E, which was replaced by ISO 14664 – 1. For class 10 000 cleanrooms (now reclassified as ISO class 7), a maximum of 10 000 particles ≥0.5 μ /feet ^{3} (352 000 m ^{3}) is permitted under static conditions. Specific requirements for viable particles or colony counts are not provided by the FDA. Guidelines published by the United States Pharmacopeia for ISO class 7 additionally require ≥0.5 airborne Colony Forming Unit (CFU)/feet ^{3} air, and ≥5 CFU/contact plate for work surfaces (17). The equipment available to monitor these parameters has been reviewed previously (13 – 15). Although the simultaneous measurement of viable and non-viable airborne particles during the set-up phase of a cellular therapy cleanroom environment has been reported (18), no relationship was drawn between non-viable particle measurements, which can be performed inexpensively in real time, and viable particle counts, which are costly and involve significant turn-around time.

Several groups have reported on airborne contamination in the setting of the operating room in relation to the risk of surgical wound contamination (9,11,12,19,20). Environmental factors such as the use of forced air-warming blowers, and operating-room personnel variables including skin exposure and the number of people in the surgical suite, have all been found to affect the risk of surgical site infections (5 – 7,19,21,22). Other areas of the hospital caring for high-risk patients with increased risk of nosocomial infection, such as

burn units and hematology/oncology wards, have put air monitoring and quality systems into place (23,24). Thus reduced airborne particulates appear to correlate with a decreased risk of nosocomial infection in high-risk patient populations.

The impetus behind FDA directives on good tissue practices in general, and environmental control and monitoring in particular, is the prevention of disease transmission by human tissue and cellular products (2). In the cellular therapy laboratory, cells and tissues collected from autologous and allogeneic donors are usually washed, concentrated and sampled for testing. Often cellular products are manipulated further, including purification of cell populations and cryopreservation. Recipients of cellular products have a wide range of illnesses and may receive immunosuppressive or even myeloablative regimens prior to receiving cellular therapy. Although endotoxin levels and Gram stain may be used as real-time product release criteria, definitive proof of product sterility typically requires a 14-day culture and is not available for cellular products that must be freshly administered. Although the majority of airborne contaminants are non-pathogenic, cellular products, particularly those that are administered intravenously, have the potential to induce severe infections. Maintaining a working environment to a well characterized and controlled standard is therefore essential to patient safety.

In addition to ensuring that cellular products are manipulated in an environment that minimizes the risk of contamination, measuring particle counts in real time can also serve as a routine diagnostic assessment of the air-handling infrastructure and current laboratory practices. Changes in facility operation, such as failure of heating ventilating and air-conditioning equipment and filter failure, and environmental disturbances such as building construction, may all result in increased particle generation. Changes in laboratory practices, or deviations from established laboratory procedures, may likewise lead to increased particulates.

As demonstrated here, our cell-processing facility was able to identify a major building equipment failure in real time that could have resulted in compromised product integrity if gone unrecognized. With modern laser-based portable particle counters, real-time analysis of daily non-viable particle counting at a variety of critical locations is simple and inexpensive. Viable particle counts, although similarly automated, use costly culture media and require incubation time and interpretation. The correspondence of viable counts to non-viable counts described here has allowed us to use non-viable particle counts for routine daily surveillance, with empirically determined sensitivity and specificity to predict meaningful levels of airborne microbial contamination. Prior to performing cell processing, non-viable particle counts are measured at three sites: the cleanroom, biosafety cabinet and testing laboratory (one observation at each site replicated eight times). The results of these measurements are downloaded from the instrument and analyzed. Data from all locations are assessed, but the action limit of 32 000 applies only to the cleanroom (the biosafety cabinet is expected to have a measurement of zero). However, non-viable particle count trends for all sites are assessed, as are temperature and humidity, which are also measured and recorded by the particle counter. If the cleanroom particle count is ≥10 000 counts/ feet ^{3} , it is operating at the expected level. A count of >10 000 but <32 000 falls with the alert limit. Activities occur as usual, but increased attention is given to subsequent determinations.

However, at count levels ≥32 000, cell-processing activities are put on hold, building facility management is notified, air-handling systems are assessed, a deep cleaning is performed according to a standard procedure, and a deviation management procedure is followed. After the cleaning, particle counts are run again and must be <32 000/feet ^{3} before normal laboratory activities can be resumed.

In addition to these measures, actual viable particle counts and settle plate readings, with speciation, are performed by an outside vendor four times annually at the time of the certification of the clean-room and biologic safety cabinets. The full working procedure formatted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (25) is available on-line (see the supplementary material to be found online at http://www.informahealthcare.com/doi/abs/10.3109/14653 249.2012.698728) and includes Reagents and Supplies, Instrumentation, Cautionary Notes, Detailed Methods, Acceptable Endpoints, Result Reporting and Quality Control sections.

In conclusion, we have described a simple and practical method for monitoring air quality in a cellular therapy laboratory, in which real-time analysis of daily non-viable airborne particle counts is used to its best advantage. An alert limit is established based on the designed rating of the cleanroom, and an action limit is derived from the empirically determined relationship between viable and non-viable airborne particles in our laboratory setting. The principal advantage of non-viable particle monitoring is the ability to identify and respond to deviations from the expected performance in real time. The implementation of this monitoring procedure has allowed us to interpret particulate date in an evidence-based manner and adjust laboratory practices to minimize the risk of compromising product sterility as a result of airborne contaminants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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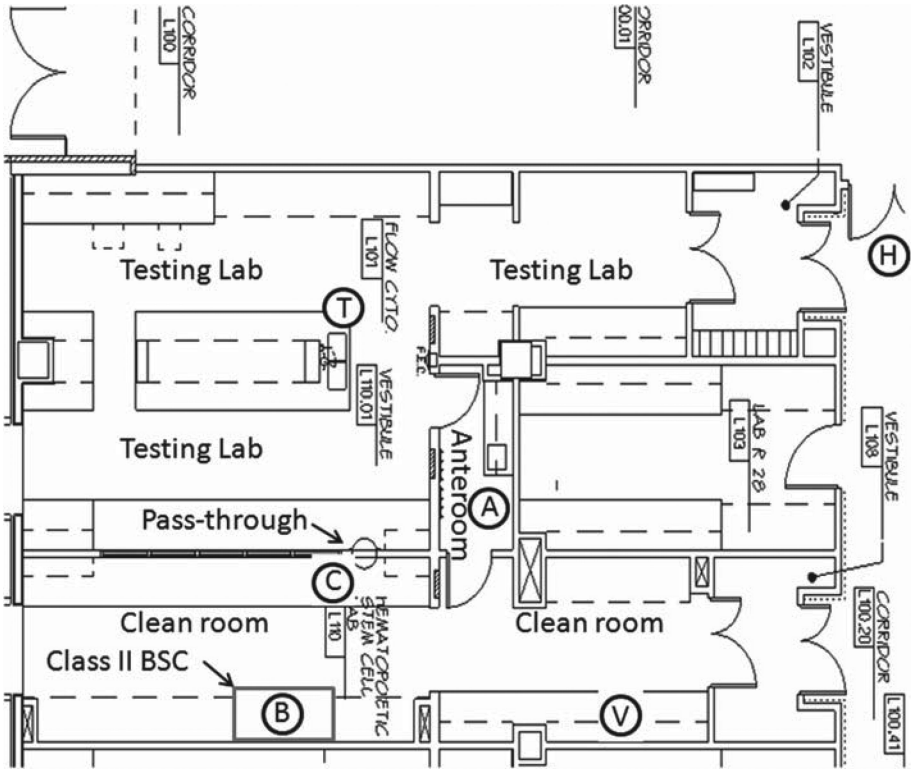

Figure 1.

Laboratory schematic showing testing sites. The sampling locations are denoted by capital letters. B, biologic safety cabinet; C and V, cleanroom (two locations); T, testing laboratory; A, anteroom; H, hallway.

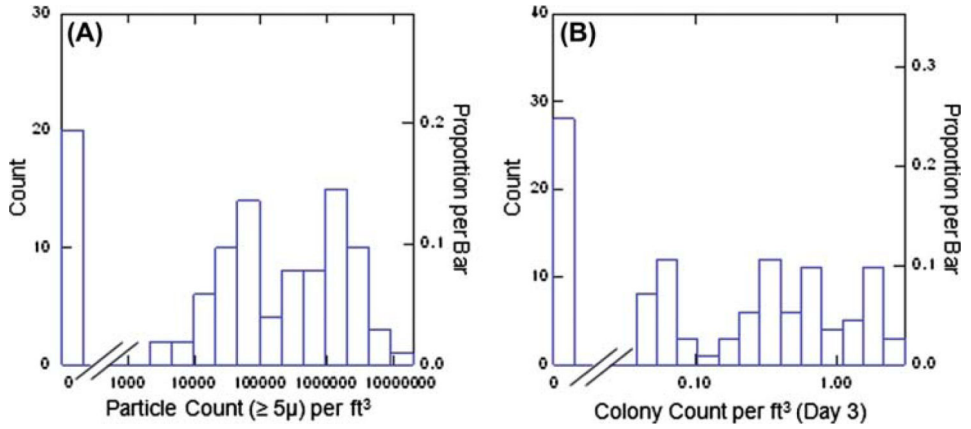

Figure 2.

Non-viable and viable airborne particle monitoring. We performed simultaneous non-viable (≥5 μ ; 227B laser particle counter; MetOne, Grants Pass, OR, USA) and viable (Biotest HYCON RCS, high-flow viable particle counter, Biotest agar strips TCI- γ ; Bioteet AG, Landsteinerster, Germany) particle measurements over a period of 1 year. A total of 20 paired measurements was made in five different laboratory locations that varied with respect to extent of air treatment (hallway, testing laboratory, anteroom between testing laboratory and cleanroom, ISO class 7 cleanroom, and class II biologic safety cabinet within the cleanroom). Non-viable particle counts (A), expressed in counts ≥5 μ /feet ^{3} of air, ranged from 0 (class II biosafety cabinet) to 10 240 000. Viable counts (B) measured in colonies/ feet ^{3} of air after 3 days of incubation, ranged from 0 (biosafety cabinet, cleanroom) to 2.5.

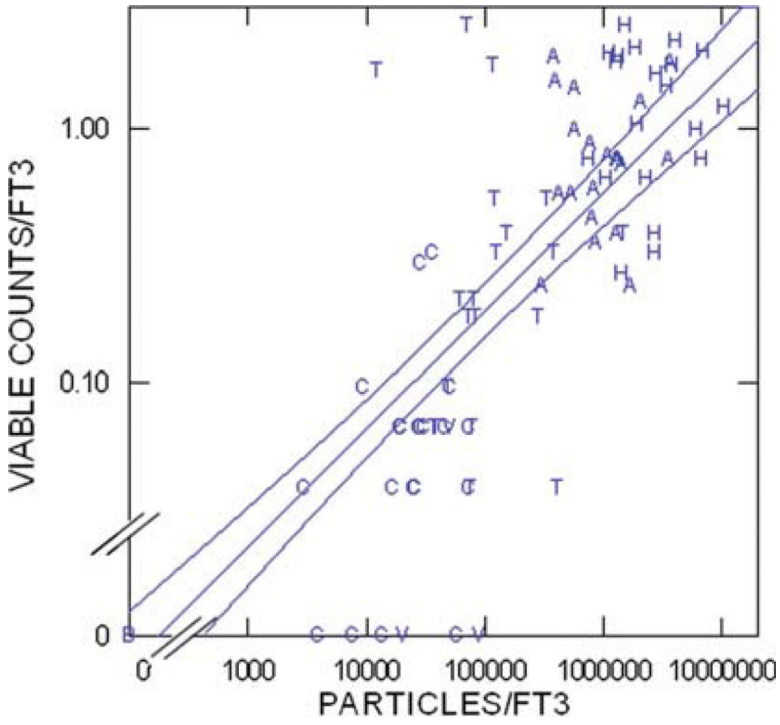

Figure 3.

Correlation of viable and non-viable particle counts. Linear regression analysis was performed on paired viable and non-viable count data. A log scale with broken axes shows zero (undetectable) counts. The line of best fit and the 95% confidence intervals are shown. Data points are shown in upper case letters corresponding to the locations shown in In the biologic safety cabinet (B), all points overlap at 0,0.

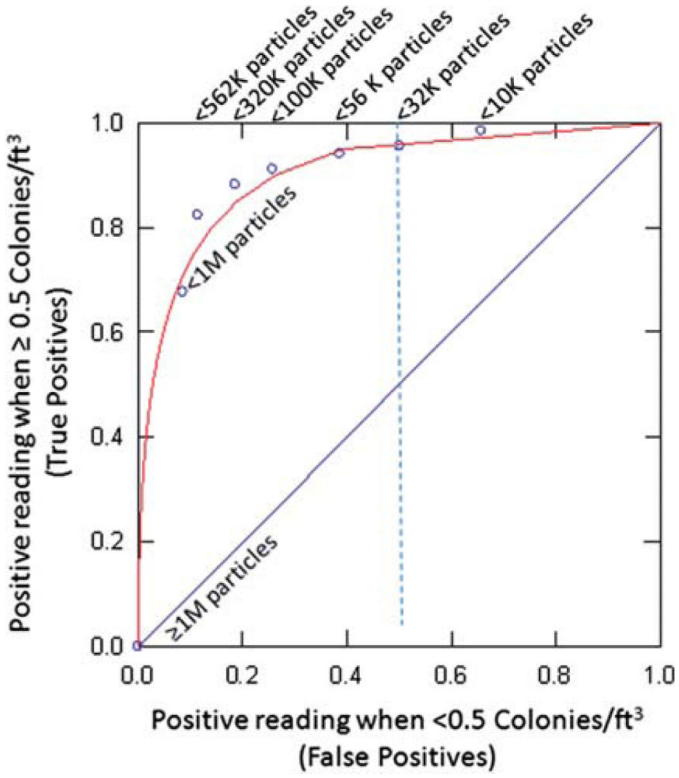

Figure 4.

ROC curve relating non-viable and viable airborne particulates sampled concurrently at the same locations. A viable particle count less than 0.5/feet ^{3} was chosen as the maximum acceptable level for an ISO class 7 cleanroom. Non-viable particle counts were binned in 1/2 log $_{10}$ increments (with two added points where data were most dense). A non-viable particle count of <32 000 particles/feet ^{3} was chosen as the action limit based on sensitivity (95.6%) and specificity (dashed line, 50%) considerations.

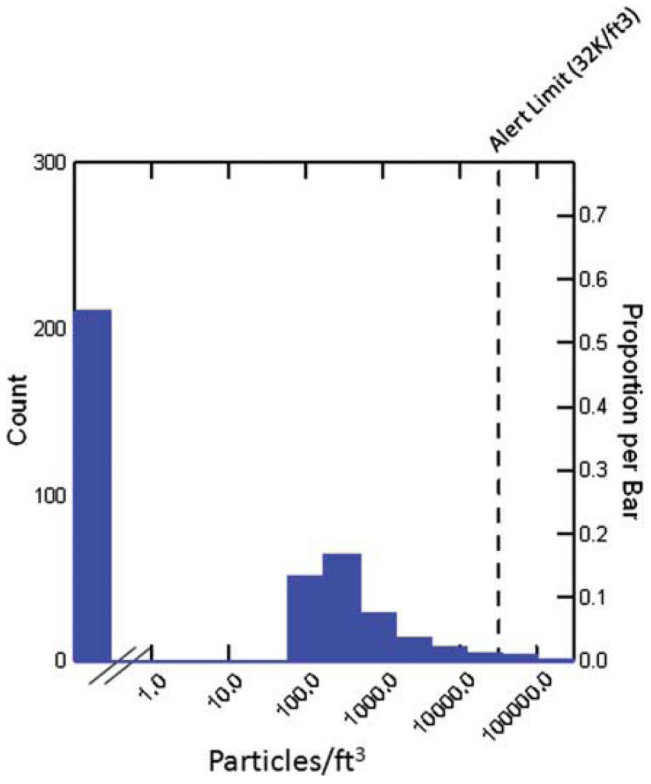

Figure 5.

Retrospective raw count data for pass-through nonviable counts, *n* = 1586 (200 determinations, eight replicates). The 99th percentile is 33 778 counts/feet ^{3} . The alert limit from ROC is 32 000 counts/feet ^{3} . At the alert limit, 50% would be false alarms, but 95.6% of all true contaminations (defined as 0.5 colonies/feet ^{3} on day 3) would be detected.

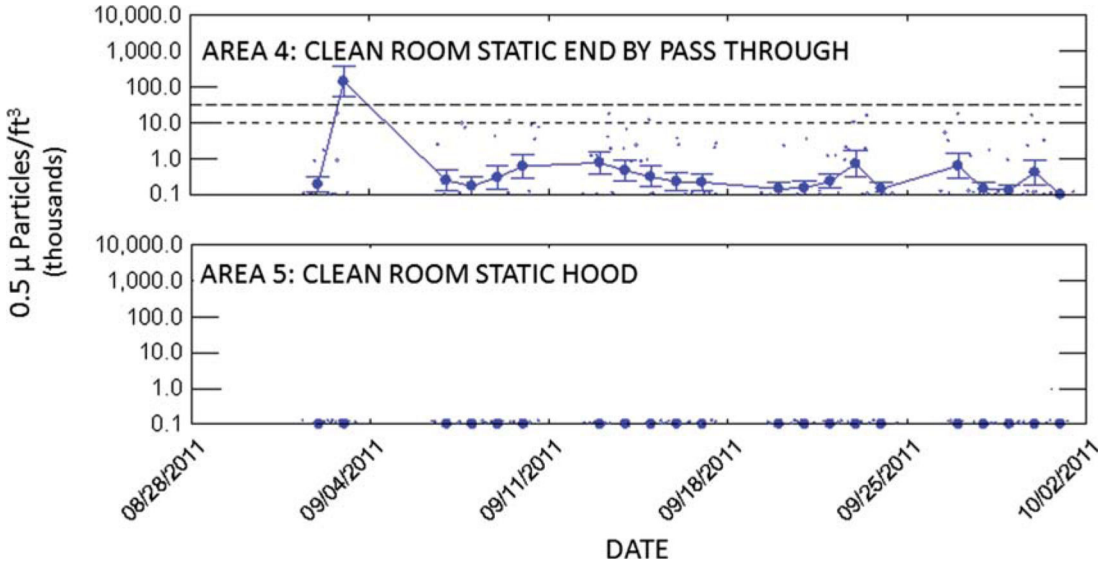

Figure 6.

Daily particle readings in the cleanroom (Figure 1C) and class II biosafety cabinet (Figure 1B). Daily particle counts were performed during working days. The bars show the standard errors associated with replicate determinations. The upper dashed line indicates the action limit for the cleanroom and the lower dashed line indicates the alert limit (10 000/feet ^{3} , lower dashed line). On 2 September 2011 the particle counts in the cleanroom exceeded the action limit (upper dashed line) and appropriate action was taken according to the protocol. Particles were not detected in the biosafety cabinet at the time of the malfunction.